

PHYLLANTHUS AMARUS ATTENUATES ETHANOL-INDUCED OXIDATIVE STRESS IN RAT INTESTINE

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ABSTRACT

In recent years, there has been escalation in alcohol abuse with an attendant increase in alcohol-related gastrointestinal tract disorders. Alcohol is known to induce a dose-dependent increase in oxidative stress. This study evaluates the protective effect of *Phyllanthus amarus* on ethanol-induced oxidative stress in the intestine of rats. Animals were pre-treated with *P. amarus* (250 and 500mg/kg/day per oral) for one week and treatment continued for additional three weeks during which the animals were simultaneously challenged with ethanol (5g/kg/day, 20% w/v orally). Oxidative stress in the intestine tissue was estimated using lipid peroxidation (LPO) and glutathione (GSH) levels, as well as superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities. Ethanol administration caused a marked ($p < 0.05$) reduction in body weight gain and organ weight albeit no significant difference in total protein content. LPO and GST levels were significantly ($p < 0.001$) increased whereas GSH, SOD and CAT levels were significantly ($p < 0.001$) decreased in the intestine of rats treated with ethanol alone. *P. amarus* administration successfully attenuated these effects, exerting a significant ($p < 0.001$) increase in GSH, SOD and CAT levels, a marked ($p < 0.001$) reduction in the levels of LPO and GST in intestine tissues. More so, treatment with 500mg/kg extract was more effective than 250mg/kg extract. In conclusion, *P. amarus* effectively protected the intestine against ethanol-induced oxidative stress by directly reducing the level of LPO and indirectly by enhancing the levels of endogenous antioxidants.

KEYWORDS: Ethanol; Oxidative Stress; *Phyllanthus amarus*; Intestine; Attenuates

INTRODUCTION

Alcohol beverages have been used and abused by a large number of individuals since the dawn of history. While light to moderate amounts of alcohol may have beneficial effects for cardiovascular health (Zakhari 1997), chronic consumption of alcohol can result in spectrum of abnormalities in many organ systems (Lindros 1995; Epstein 1997; Oscar-Berman *et al* 1997). Among the many organ systems that mediate alcohol's effects on the human body and its health, the GIT and the liver play crucial roles (Bode and Bode 1997). The GIT is the primary site of alcohol absorption into the systemic circulation while the liver is the primary site of alcohol metabolism in the mammalian body (Lieber 2000). To a lesser extent, alcohol can also be oxidized (first pass metabolism) and produced in the GIT (Bode and Bode 1992). Although metabolism of alcohol in the GIT is quantitatively much lower compared to the liver, it is of importance because it affects the systemic availability of alcohol, local production of toxic acetaldehyde and ROS, which have been implicated in the pathogenesis of tissue injury (Bode and Bode 1997; Lieber 1997).

Chronic alcohol consumption can exert deleterious effects on the structures and functions of all parts of the GIT (Bode and Bode 1997). The direct contact of alcohol with the GIT mucosa may elicit several metabolic changes among which is the induction of ethanol-inducible cytochrome P450 (CYP2E1) with an attendant generation of toxic acetaldehyde and reactive oxygen species (ROS) (Hakkak *et al* 1996). These metabolites are capable of depleting endogenous antioxidant status and may thus disturb the integrity of the mucosal epithelium (Zima *et al* 2001; Wu and Cederbaum 2003). This, may in concert with other mechanisms, contribute to the development of alcohol-related pathologies of the GIT such as gastrointestinal bleeding, inflammation, ulceration, diarrhoea and less well-recognized influences on other diseases especially cancer (Bode and Bode 1997; Zima *et al* 2001; Crabb *et al* 2004). More so, alcohol-induced mucosal injuries, especially in the vulnerable duodenum, have been reported to

render the gut more permeable to bacterial toxins. These toxic substances have been implicated in the pathogenesis of alcohol-induced damage to the liver and other organs (Bode and Bode 1997). In addition, alcohol-induced functional alterations and mucosal damage have been reported to cause disturbances in the digestion, absorption and assimilation of other nutrients into the body, thereby contributing to the malnutrition and weight loss often observed in alcoholics (Thomson and Pratt 1992; Bode and Bode 1997; Lieber 2000).

Phyllanthus amarus Schum and Thonn, family Euphorbiaceae, is a small erect, annual herb that grows 30 to 40cm in height (Khanna *et al* 2002; Jaleel *et al* 2007). It is indigenous to the rainforest of the Amazon and other tropical areas throughout the world. *P. amarus* is locally referred to as Eyin-olubisowo or dobiosowo (Yoruba), Geeron-tsuntsaye (Hausa), Buchi oro (Ibo), Oyomokeso (Efik). It is widely used in the folk medicine of different countries (Calixto *et al* 1998). It is a well known hepatoprotective (Sane *et al* 1995) and antiviral (Thyagarajan *et al* 1990) agent. Some other studies also reported that *P. amarus* possesses antioxidant, anti-diabetic (Rapheal *et al* 2002), radioprotective (Kumar and Kuttan 2004) and antimutagenic (Rapheal *et al* 2002) properties. Recent study in our laboratory (Odetola and Akojenu 2000) showed that *P. amarus* is a formidable source of protection against castor oil-induced diarrhoea in experimental model.

On the basis of these considerations, the aim of the present study was to evaluate the protective effect of *P. amarus* on ethanol-induced oxidative stress in the intestine. In this study, *P. amarus* was administered prior and during ethanol intoxication in order to determine preventive and protective effects on ethanol-induced oxidative injury. Our hypothesis is that the use of herbal medicine may make it possible to prevent and alleviate the incidence of alcohol-induced gastrointestinal disorders via inhibition of oxidative stress.

MATERIALS AND METHODS

Preparation of *P. amarus* Extract

Aerial parts (stem and leaves) of *P. amarus* were collected in April 2005 from Ado-Ekiti town, Nigeria. It was authenticated at the Forestry Research Institute Nigeria (FRIN), Ibadan and a voucher specimen (no FHI 107623) has been deposited at the Forestry Herbarium Ibadan (FHI). The leaves were air-dried at room temperature, powdered and 100g were extracted with 500 ml of 80% methanol by stirring overnight at room temperature (25°C). The mixture was then centrifuged at 2,500 rpm to separate the supernatant, the supernatant was filtered (Whatman No 3) and evaporated to dryness at 45°C with a rotary evaporator. The extract was dissolved in water at a concentration of 3g/100ml to form *P. amarus* solution.

Phytochemical studies carried out with *P. amarus* aerial parts have demonstrated the presence of many classes of constituents, such as tannins (phyllanthusin D, repandusinic acid, 1,6-digalloyl glucopyranose, geraniin, amariin, furosin, geraniinic acid B, amariinic acid, amarulone, corilagin, furosin and elaeocarpusin) (Foo and Wong 1992; Foo 1993; Foo 1995), alkaloids (securinine, norsecurinine, epibubbialine and isobubbialine) (Houghton *et al* 1996) phenolics (gallic acid and 4-O-galloylquimic acid) (Foo 1993) and flavonoids (catechins, gallocatechin, quercetin, rutin and quercetin-3-O-glucopyranoside) (Morton 1981; Foo 1993), polyphenols (ellagic acid, phenazine and phenazine derivatives) (Foo 1993)

Animals and Treatments

A total of 30 male albino-Wistar rats weighing 180 ± 5 g were used for the experiment. The animals were purchased from the Institute of Medical Research and Training (IMRAT), University College Hospital, Ibadan. They were kept in specially designed, well ventilated plastic cages under standard conditions and were maintained on normal laboratory chow (Bendel Feed and Flour Mill Ltd, Edo-State) and water *ad libitum*. All animal experiments were conducted without anaesthesia and according to the *Ethical Norms on Animal Care and Use* approved by IMRAT and Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan.

After two weeks of acclimatization, the rats were randomly divided into five treatment groups of six animals each (Table 1). The PA, ETPA and ETPAPA groups were pre-treated with *P. amarus* extract for one week and treatment continued for additional three weeks during which ET, ETPA and ETPAPA groups were treated with ethanol solution while the normal and PA groups were treated with isocaloric glucose solution. *P. amarus* extract and ethanol were administered by gavages respectively, two hours apart and once daily. At the end of the experimental

period of four weeks, the final body weight of the rats was determined and they were fasted 12 hours prior to when they were sacrificed by cervical dislocation.

Collection and Preparation of Tissue

All rats were carefully dissected and the proximal portion (15cm) of the small intestine was excised, washed and flushed thoroughly with ice-cold physiological saline, blotted on filter paper, weighed, and homogenized in ice-cold 1.15% KCl (1g/3ml) in a Potter-Elvehjem type homogenizer. The homogenates were centrifuged at 5000 rpm for 15min at 4°C. Aliquots of the supernatant were used for the following biochemical assays.

Biochemical analysis

The total protein content of the aliquot was determined by the method of Lowry *et al* (1951), using bovine serum albumin as standard.

Lipid peroxidation: Lipid peroxidation was determined by estimating malondialdehyde (MDA) content using the thiobarbituric acid test (Beuge and Aust (1978). Briefly, the stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25 N hydrochloric acid and 2-thiobarbituric acid 0.37% (w/v) in 0.25 N hydrochloric acid. One volume of the test sample and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15min on a boiling water bath. After cooling on ice the precipitate was removed by centrifugation at 4000rpm for 15min and absorbance of the supernatant was measured at 532 nm against blank containing all the reagents except test sample. TBARS were calculated using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ and expressed as $\mu\text{M MDA (g tissue)}^{-1}$.

Reduced glutathione: Reduced glutathione level was assayed by the method of Jollow *et al* (1974) based on the reaction with 5,5'-dinitro bis (2-nitrobenzoic acid) (DTNB). Briefly, 1.0ml of sample (10%) was deproteinized by adding an equal volume of 4% sulfosalicylic acid. The sample was kept at 4°C for at least 1 hour and then centrifuged at 4200 rpm for 15min at 4°C. 0.5ml of the resulting supernatant was added to 4.5ml of DTNB in phosphate buffer (0.1 M, pH 7.4). The yellow colour developed was read immediately at 412 nm on a spectrophotometer. A blank was prepared by adding 0.5ml of 4% sulfosalicylic acid to 4.5ml DTNB.

Antioxidant enzyme activity: The activity of SOD (EC 1.15.1.1) was determined spectrophotometrically at 480 nm by the epinephrine method (Misra and Fridovich 1972) and expressed in units of enzyme activity per milligram protein. CAT (EC 1.11.1.6) activity was estimated spectrophotometrically at 570 nm by the method of Sinha (1971) by measuring the rate of decomposition of hydrogen peroxide (H_2O_2). The method of Habig *et al* (1974) was followed to assay the activity of GST (EC 2.5.1.18) by monitoring the appearance of the conjugated complex of GSH and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm.

Statistical analysis

All results are presented as mean $\pm$ SD, and differences were analyzed by one-way analysis of variance (ANOVA) followed by Fischer's LSD post hoc test using SPSS 11 software (SPSS Inc, Chicago). When appropriate, Independent sample t test was done to evaluate the significance of the differences between means. Statistical significance was considered at $p < 0.05$.

RESULTS

During the experiment, the body weight increased in all experimental rats (Table 2). However, the increase was significant in the normal, PA, ETPA and ETPAPA groups ($p < 0.01$, $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively). Treatment with ethanol alone significantly ($p < 0.05$) reduced the weight of the intestine although no significant decrease in the total protein content. Co-treatment of varied doses of *P. amarus* with ethanol had no significant effect on intestine weight and total protein content albeit a general improvement in these parameters relative to ET-treated rats (Table 2).

The level of lipid peroxidation (LPO) and reduced glutathione (GSH) are presented in Table 3. The level of lipid peroxidation, as assessed by malondialdehyde (MDA), in the intestine was significantly ($p < 0.001$) increased in ET-treated group relative to normal, ETPA and ETPAPA groups (Figure 1). A significant ($p < 0.001$) decrease in LPO

level was observed in ETPAPA-treated group as compared with ETPA-treated group. There was a marked ($p < 0.001$) decrease in the intestine GSH level of ET-treated group compared to normal, ETPA- and ETPAPA-treated groups as well as a marked ($p < 0.001$) increase in ETPAPA-treated group relative to ETPA group.

The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione-S transferase (GST) are presented in Table 4. The activity of SOD in the intestine as shown in Table 4 followed a similar pattern as in reduced GSH level (Table 3). The activity of CAT in the intestine was significantly ($p < 0.001$) reduced in ET-treated group relative to normal and ETPAPA-treated groups but not significant relative to ETPA-treated group. More so, CAT activity was significantly ($p < 0.01$) enhanced in ETPAPA-treated group compared to ETPA-treated group. The activity of GST in the intestine increased significantly ($p < 0.001$) in ET-treated group relative to normal, ETPA and ETPAPA groups. A marked ($p < 0.05$) decrease in GST activity was observed in ETPAPA-treated group relative to ETPA-treated group.

DISCUSSION

There is increasing evidence that chronic alcohol consumption can exert deleterious effects on the structures and functions of all parts of the GIT via oxidative stress (Bode and Bode 1997). The direct contact of alcohol with the GIT mucosa has been reported to disturb the integrity of the mucosal epithelium leading to mucosal injuries particularly in the duodenum (Bode and Bode 1997). This may interfere with digestion and absorption of essential nutrients in the GIT (Lieber 2003). Poor nutritional status, as a consequence of alcohol-induced primary and secondary malnutrition, has been reported to contribute to loss in body weight and the development of other alcohol-related pathologies (Lieber 2003; Poschl *et al* 2004). Hence, in our study, there was a significant increase in body weight gain in all animals except the ET-treated group. More so, a marked reduction in organ weight was observed, albeit no significant difference in total tissue protein in ET-treated group relative to the normal and other groups.

Treatment with varied doses of *P. amarus* markedly restored the ethanol-induced alterations in body and organ weights. These positive effects may be attributed to the protective effect of *P. amarus* extract on the GIT. A similar observation has been reported by Odetola and Akojenu (2000).

It is a well established fact that alcoholic patients and experimental animals exposed to chronic ethanol display biochemical signs of oxidative damage (Wu and Cederbaum 2003; Rukkumani *et al* 2004; Uzun *et al* 2005). GIT metabolism of alcohol is quantitatively much lower compared to the liver but it is of importance because it plays a major role in the local accumulation of toxic acetaldehyde and ROS, implicated in mucosal injury (Lieber 1997; Seitz and Oneta 1998). Excessive generation of ROS, as a result of CYP2E1 induction in the GIT after chronic ethanol consumption, has been reported to play a major role in peroxidative damage of epithelial membrane lipids (Albano and Clot 1996; Hakkak *et al* 1996). In our present study, there was a marked increase in the level of LPO by 284% in the intestine of ET-treated animals relative to control animals. Other authors have reported similar observations (Bode and Bode 1992; French *et al* 1993).

The antioxidant defence system protects the aerobic organism from the deleterious effects of ROS (Halliwell 1999). Glutathione (GSH), an important endogenous non-enzymatic antioxidant is involved in the protection against free radicals and other toxic compounds such as acetaldehyde (Rukkumani *et al* 2004). Glutathione-S transferase (GST) is a key component of cellular antioxidant defenses, offering protection against cell damage (Dinkova-Kostova and Talalay 1999). Catalase (CAT), which acts as preventative antioxidant and SOD, a chain-breaking antioxidant, play a vital role in protection against the deleterious effects of LPO (Rukkumani *et al* 2004; Uzun *et al* 2005). In our study, chronic ingestion of ethanol challenges the antioxidant defence system by causing a remarkable decrease in the level of GSH, SOD and CAT by 73.29%, 53.43% and 63.04% respectively and a marked increase in GST activity by 85.58%. These findings are indicative of an obvious change in the oxidant-antioxidant balance in the intestine of rats, which may culminate in gastric and duodenal ulcer (Poschl *et al* 2004). Some investigators have reported similar findings in the level of GSH, SOD and CAT (Pronko *et al* 2002; Ozaras *et al* 2003; Rukkumani *et al* 2004; Uzun *et al* 2005). The observed depletion in GSH level may be attributed, in part, to increase utilization to counteract ROS and lipid peroxidative products and toxic acetaldehyde. In addition, alcohol itself has been reported to inhibit the biosynthesis of new GSH (Lieber 2003). The inverse correlation between ethanol-induced LPO and the activities of SOD and CAT is indicative of an overwhelming oxidative stress which is further corroborated by heightened GST activity.

The fact that ethanol intoxication can induce a state of oxidative stress implies that administration of antioxidants before or after ingestion of alcohol could possibly prevent or diminish the processes of alcohol-induced oxidative damage in the tissues (Molina *et al* 2003; Ozaras *et al* 2003; Rukkumani *et al* 2004). The results obtained in this study agree with this hypothesis. Treatment of ethanol-intoxicated rats with varied doses of *P. amarus* extract (250 and 500 mg/kg) significantly reduced the level of LPO by 28.22% and 61.81% respectively. The antioxidant defence system was positively modulated. The ethanol-induced decrease in the levels of GSH, SOD and CAT were significantly restored in the ETPA (250mg)-treated group by 91.07%, 51.37% and 12.52% respectively, and in the ETPAPA (500mg)-treated group by 160.71%, 103.29% and 39.66% respectively relative to the ET-treated group. In addition, the ethanol-induced elevation of GST activity was significantly reduced in the ETPA- and ETPAPA-treated animals by 29.57% and 44.36% respectively relative to the ET-treated animals.

Phytochemical analysis of the extract show that *P. amarus* contains several bioactive ingredients such as benzenoids, tannins and flavonoids of proven antioxidant activity *in-vitro* (Joy and Kuttan 1995; Calixto *et al* 1998). Thus, the observed protection against LPO may be attributed to the antioxidant phytochemicals present in *P. amarus* (Sane *et al* 1995; Raphael *et al* 2002). More so, the restoration of the antioxidant defence system in this study may be due, in part, to the antioxidant-sparing actions of the phytochemicals, particularly, the polyphenolic compounds (Sane *et al* 1995; Calixto 1998; Raphael *et al* 2002). Apart from antioxidant sparing action, polyphenolic compounds have been reported to enhance the biosynthesis of reduced GSH by up-regulating the expression of γ -glutamylcysteine synthetase, the rate-limiting enzyme in the biosynthesis of reduced GSH (Moskaug *et al* 2005).

CONCLUSIONS

In conclusion, our study shows that ethanol intoxication resulted in an increased oxidative stress in rat intestine. It also demonstrated that administration of *P. amarus* extract prior and during ethanol intoxication may exert a marked protective effect on ethanol-induced oxidative damage. This finding is plausible as it can be seen from the positive effects of the extract on the parameters considered above in the PA-treated group compared to the normal group. Our study, therefore, suggests a role for *P. amarus* as a safe, cheap and effective phytotherapy in the prevention and management of ethanol-induced oxidative damage, a causative factor in duodenal ulcer and other GIT disorders.

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Table 1 Experimental design

Group	Treatment
Control	Isocaloric glucose solution
ET	Ethanol solution (5g/kg/day)
PA	<i>P. amarus</i> extract (250mg/kg/day) and Isocaloric glucose solution
ETPA	<i>P. amarus</i> extract (250mg/kg/day) and ethanol solution (5g/kg/day)
ETPAPA	<i>P. amarus</i> extract (500mg/kg/day) and ethanol solution (5g/kg/day)

Notes: $n = 6$ for each treatment group.

Table 2 Effects of ethanol and *Phyllanthus amarus* treatments on the body weight, intestine weight and total protein content of rats.

Experimental	Body Weight			Intestine/body weight	Total Protein Content
Group	Initial (g)	Final (g)	% Change	ratio ($\times 10^{-2}$ g)	(mg/ml)
Control	180.00 $\pm$ 0.00	216.67 $\pm$ 16.23	20.37 #	3.01 $\pm$ 0.82	40.14 $\pm$ 9.46
ET	180.00 $\pm$ 8.16	183.33 $\pm$ 21.91	1.89	2.27 $\pm$ 0.20 * ^a	38.68 $\pm$ 11.12
PA	180.00 $\pm$ 0.00	223.33 $\pm$ 8.16	24.07 ‡	2.75 $\pm$ 0.30	44.32 $\pm$ 5.88
ETPA	180.00 $\pm$ 0.00	196.67 $\pm$ 8.16	9.26 #	2.60 $\pm$ 0.54	45.12 $\pm$ 7.94
ETPAPA	180.00 $\pm$ 0.00	206.67 $\pm$ 24.22	14.82 *	2.39 $\pm$ 0.37	41.91 $\pm$ 6.10

Notes: Values are means $\pm$ SD. $n = 6$ for each treatment group. Body weight data were analyzed by Independent sample t test and comparison was done between initial weight and final weight. Intestine weight and total protein content were analyzed by one-way analysis of variance (ANOVA) followed by Fischer's LSD post hoc test. a = Control vs. other groups; b = ET vs. ETPA and ETPAPA; c = ETPA vs. ETPAPA; * = $P < 0.05$; # = $P < 0.01$; ‡ = $P < 0.001$.

Table 3 Effects of *Phyllanthus amarus* extract on the levels of lipid peroxidation and reduced glutathione in the intestine of ethanol-exposed rats.

Experimental group	MDA content ($\mu\text{g/g}$ tissue)	Glutathione content ($\mu\text{g/g}$ tissue)
Control	14.07 ± 1.93	6.29 ± 0.43
ET	$54.04 \pm 4.15 \ddagger^a$	$1.68 \pm 0.32 \ddagger^a$
PA	$10.13 \pm 1.23 *^a$	$6.80 \pm 0.36 *^a$
ETPA	$38.79 \pm 3.92 \ddagger^b$	$3.21 \pm 0.26 \ddagger^b$
ETPAPA	$20.64 \pm 1.81 \ddagger^{bc}$	$4.38 \pm 0.25 \ddagger^{bc}$

Notes: Values are means $\pm$ SD. $n = 6$ for each treatment group. Data were analysed by one-way analysis of variance (ANOVA) followed by Fischer's LSD post hoc test. a = Control vs. other groups; b = ET vs. ETPA and ETPAPA; c = ETPA vs. ETPAPA; * = $P < 0.05$; # = $P < 0.01$, $\ddagger = P < 0.001$.

Table 4 Effects of *Phyllanthus amarus* extract on the activities of superoxide dismutase, catalase and glutathione-S transferase in the intestine of ethanol-exposed rats.

Experimental group	Superoxide dismutase Activity (U/mg protein)	Catalase Activity (Kat f)	Glutathione-S transferase Activity (μg/min/mg protein)
Control	3.93 ± 0.16	28.52 ± 0.64	2.15 ± 0.42
ET	1.83 ± 0.22 ‡ ^a	10.54 ± 1.92 ‡ ^a	3.99 ± 0.81 ‡ ^a
PA	4.28 ± 0.28 * ^a	30.05 ± 1.72	1.99 ± 0.26
ETPA	2.77 ± 0.28 ‡ ^b	11.86 ± 1.57	2.81 ± 0.40 ‡ ^b
ETPAPA	3.72 ± 0.17 ‡ ^{bc}	14.72 ± 1.48 ‡ ^b # ^c	2.22 ± 0.40 ‡ ^b * ^c

Notes: Values are means ± SD. *n* = 6 for each treatment group. Data were analysed by one-way analysis of variance (ANOVA) followed by Fischer's LSD post hoc test. a = Control vs. other groups; b = ET vs. ETPA and ETPAPA; c = ETPA vs. ETPAPA; * = P<0.05; # = P<0.01, ‡ = P<0.001. Catalase feiahigkeit or "Kat f" is equivalent to μmole H₂O₂ consumed/min/mg protein.